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STUDY OF THE PATHOGENICITY OF AEROMONAS HYDROPHILA FOR MAN

Annual Report

Herbert L. DuPont, MD

August 15, 1983

Supported by

U.S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

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University of Texas Health Science Center at Houston

Houston, Texas 77030

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A pool of volunteers from the Houston Community were attracted to our institution for efficient study in the hospital. Three strains of <u>A. hydrophila</u> were selected for study in volunteers based on: recovery from patients in well defined studies; laboratory studies of the presence of virulence properties; recovery from selective media; and antibiograms. The test strains were fed to 5 groups of volunteers in doses ranging from 10^4 to 10^7 viable cells. Transient excretion of the test strain rarely occurred in any group and despite the passage of several soft and rare watery stools by four volunteers frank illness did not develop. We are		

Summary

A pool of volunteers from the Houston Community were attracted to our institution for efficient study in the hospital. Three strains of A. hydrophila were selected for study in volunteers based on: recovery from patients in well defined studies; laboratory studies of the presence of virulence properties; recovery from selective media; and antibiograms. The test strains were fed to 5 groups of volunteers in doses ranging from 10^4 to 10^7 viable cells. Transient excretion of the test strain rarely occurred in any group and despite the passage of several soft and rare watery stools by four volunteers frank illness did not develop. We are currently looking at the development of humoral antibodies in the volunteers and are feeding the test strains in a dose of 10^8 viable cells.

Forward

For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.

TABLE OF CONTENTS

	Page
Nature of Work Being Reported	5
Statement of Problem	5
Background	5
Approach to Problem	6
Results	7
Discussion and Conclusions	8
Tables	9
Literature Cited	14
Appendices	15

TABLES AND APPENDICES

Table 1. Historical Considerations of A. hydrophila Test Strains

Table 2. Virulence Properties of A. hydrophila Test Strains

Table 3. Antimicrobial Susceptibility of A. hydrophila Test Strains

Table 4. Reactions in Volunteers During 6 Days After Ingestion of 10^4 A. hydrophila Cells

Table 5. Reactions in Volunteers During 5 Days After Ingestion of $10^6 - 10^7$ A. hydrophila Cells

Appendix A. Examination for Participating Students

Appendix B. "Help Stamp Out Turista" Recruiting Poster

Nature of Work Being Reported

The present report deals with 6 1/2 months of a total of 12 months concerning contract No. C-3024. The project thus far has focused on the establishment of the volunteer pool, the accumulation and evaluation of test strains and the administration of two doses of the strains to volunteers.

Statement of Problem

Approximately 50% of acute diarrhea is associated with unformed stools negative for known enteric agents. The fact that antimicrobials successfully treat or prevent a proportion of the illness suggests that poorly derived bacterial agents will prove to be important causes. A. hydrophila has recently been shown to be one of the most common agents identified in cases of diarrhea occurring in Thailand, Western Australia, and Canada. The present study is designed to determine the pathogenesis of A. hydrophila for man.

Background

Strains identified in the genus *Aeromonas* have been associated with selected cases of diarrhea for the past thirteen years (1-5). Echeverria recently found that A. hydrophila commonly was associated with acute diarrhea among U.S. Peace Corps workers in Thailand (5). These strains were also found to be present in stools of high percentage of asymptomatic persons in Thailand and it was found to be commonly present in a variety of water sources.

Most strains of A. hydrophila strains demonstrate cytotoxic activity in several tissue-cultured cell lines (6,7). The cytotoxin is heat-labile and non-dialyzable. Cytotoxin activity appears to be a stable property and may not be associated with a plasmid (7). Cytotoxin activity correlates with enterotoxin activity. Evidence has been put forth that strains from diarrhea cases are cytotoxigenic while those associated with no symptoms are characteristically cytotoxin-negative (7), while a second study failed to see this difference (8).

The illness associated with *Aeromonas* is usually of short duration with low grade fever. However, a dysentery-like illness does apparently occur (4). Although a statistical relationship exists between illness and fecal recovery of A. hydrophila, definite proof of the pathogenicity of the organism for man is lacking. Oral administration of whole cultures (10^9) of cytotoxic A. hydrophila failed to cause diarrhea in rhesus monkeys (8). It remains for volunteer studies to establish disease producing capabilities.

Approach to Problem

The test strains:

During the 6 1/2 months of study covered thus far in the contract we have obtained the A. hydrophila strains to be tested (table 1), tested them for virulence properties (table 2) and for growth on differential media, performed antimicrobial susceptibility testing of the strains (table 3) and tested two strains at a dose of 10^4 viable cells (table 4) in volunteers and three at a higher dose of $10^6 - 10^7$ (table 5).

Two strains tested (6 Y and 518) were originally recovered from asymptomatic persons (one in Thailand, the other in Western Australia). The third strain evaluated (3647) was previously recovered from a patient in Western Australia with acute diarrhea. The fourth test strain (BW64), isolated from a U.S. adult with acute diarrhea acquired in Mexico July 1983 has not yet been fed to volunteers.

All three test strains fed to volunteers have been found to possess virulence properties including production of hemolysin, cytotoxin, enterotoxin and strain 6 Y has been shown to be invasive in the rabbit ileal loop (Dammin, G., personal communication).

Challenge Studies:

After volunteers successfully passed a history and physical examination, a complete laboratory screening was performed which included EKG, chest x-ray, blood chemical analysis, and a complete blood count and differential. Volunteers were explained the study twice and they were required to successfully take a written examination on the details of the study (Appendix A). Volunteers passing the various tests were then admitted to Methodist's Hospital General Clinical Research Center on a Friday. The challenge inoculum was then administered on Friday afternoon. Volunteers were maintained in the hospital for 5-7 days being evaluated multiple times each day for the occurrence of clinical symptoms. All stool samples passed were collected. Stool weights were carried out and each specimen was cultured on media containing DNAase and MacConkey agar.

Sera were collected prior to challenge and one month later for humoral antibody development. When illness occurred, it was scheduled to perform as many procedures to determine pathogenesis of infection that would be accepted to the volunteer. This might include small bowel intubation and biopsy and sigmoidoscopy.

Administration of Inoculum:

Volunteers were fasted for ninety minutes before and after receiving the test strain. At the time of challenge, the volunteers swallow 150 ml of water containing 2 grams of NaHCO_2 . The inoculum, diluted into 1 ml, is then added to 30 ml of the bicarbonate suspension and fed to volunteers.

Results

The Test Strains:

Initially; we needed to collect representative strains of A. hydrophila for laboratory testing to determine which strains would be further studied in volunteers. Four strains of approximately 10 received were selected for further study (table 1). Dr. Peter Echeverria supplied through Dr. Formal strain 6 Y which has been the best characterized in the laboratory. It showed cytotoxicity and invasiveness for epithelial cells as studied in a 7-hour rabbit loop. In the rabbit model, strain 6 Y produced necrosis, polymorphonuclear leukocyte exudation and bacterial adherence to the mucosal surface. Invasion was noted to be limited to focal areas of the epithelium with local multiplication. Strain 518, obtained from an asymptomatic individual in Western Australia studied by Dr. Michael Gracey similarly produced characteristic Aeromonas virulence properties (table 2). No laboratory differences were seen among the strains selected by us for study in volunteers. The major difference between the strains was their association with diarrhea or not. While the original contract outlined studies comparing a cytotoxigenic positive and a toxin negative strain to confirm the importance of the virulence property as a determinate of pathogenicity for man we elected to show that Aeromonas could in fact induce illness before studying the importance of one or more virulence properties.

Table 3 shows the in vitro susceptibility of the three test strains fed to volunteers. We have planned to give an antimicrobial agent to volunteers excreting the challenge strain prior to discharge from the hospital to prevent the possibility of organism transmission in the community. Trimethoprim/Sulfamethoxazole (TMP/SMX) is the agent to be used in the following dose; TMP 160 mg + 800 mg SMX twice daily for 3-5 days.

Various selective media were tested for their potential for identification of the Aeromonas test strains (9). Media tested included: TCBS, MacConkey, Salmonella-Shigella, Tergitol 7 with 1% TCC, DNAase with bile salts and crystal violet and xylose agar. TCBS and xylose agars were found to not be suitable for growth of the test strains of A. hydrophila. DNAase was felt to be the optimal media when combined with the Oxidase test. It was noted that a clearly visible zone developed around DNAase positive colonies.

We then employed DNAase medium followed by Oxidase testing in the evaluation of 200 stool specimens from patients with diarrhea in Mexico during the summer of 1983 and were able to recover 7 strains of A. hydrophila on the primary DNAase agar plates. Stools from these patients are being studied to determine if cytotoxin can be directly assayed in stool.

The Volunteer Population:

A formal recruiting protocol was established to ensure a continuous pool of volunteers. The first step was to advertise the study in local university newspapers and The Houston Chronicle (city-wide circulation). In addition posters were distributed to 10 local universities and 6

institutions throughout the Texas Medical Center (Appendix B).

The advertisements instructed potential volunteers to call a phone number to learn about the study from a recruiter. Approximately 100 responses were received, 55 of which indicated an interest in further screening. They were asked to come to The Methodist Hospital to hear the study explained again, and to be scheduled for medical screening. During medical screening a medical history form was completed for 36 individuals and was reviewed by a physician, following which volunteers were sent to the clinic for chest x-ray, EKG, and serological testing. From this group, 23 suitable volunteers were selected. The final group of volunteers was chosen on the basis of medical history, screening results, and availability. We feel that a mechanism has been developed whereby a continual source of volunteers for these studies will be available.

Results of the A. hydrophila Feeding Study:

Tables 4 and 5 show the results of our challenge studies where five groups of four volunteers swallowed 10^4 or $10^6 - 10^7$ viable cells of one of three strains. Passage of soft stools commonly occurred but no one was considered to have developed a significant diarrheal illness. The test strains were rarely recovered from stool. We are currently planning on administering the strains in a dose of 10^8 cells.

Discussion and Conclusions

The following things have been accomplished in the first 6 1/2 months of the present contract:

1. Test strains have been selected and tested for virulence and growth properties and antibiograms.
2. A volunteer pool has been identified in Houston for these and future studies.
3. No illness has resulted when $10^4 - 10^7$ viable A. hydrophila cells were administered to volunteers.

We are currently planning to admit three groups of volunteers to the hospital to administer the test strains in a dose of at least 10^8 cells. Also, serologic studies are underway to document the occurrence of immunologic exposure to the strains and their virulence properties. We have not yet shown that A. hydrophila is a potential human pathogen.

Table 1. Historical Considerations of A. hydrophila Test Strains

Code Number	History
6 Y	Isolated from an asymptomatic Peace Corps volunteer collected after 5 weeks in Thailand.
518	Isolated from an asymptomatic individual from Australia.
3647	Isolated from a patient with diarrhea in Australia.
BW64	Isolated July 1983 from a patient with diarrhea after 3 weeks in Mexico. Symptoms included dysentery, fever, and bloody stools. Patient improved with TMP/SMX therapy.

Table 2. Virulence Properties of A. hydrophila Test Strains

Code Number	Beta Hemolysis Rabbit RBC's Sheep RBC's		Rabbit Ileal Loop Reaction Whole Organisms Supernatants		Suckling Mouse Assay	YAC Assay	Guinea Pig Eye Test
6 Y	4+	4+	+	+	+	+(100)%	-
518	4+	4+	ND	+	ND	+(100)%	ND
3647	4+	4+	ND	ND	+	ND	ND
BW64	ND	ND	ND	ND	ND	ND	ND

ND= Not done

Table 3. Antimicrobial Susceptibility of A. hydrophila Test Strains

Code Number	Amp	Furoxone	$\frac{\text{TMP}}{\text{SMX}}$	TMP	Tetra	Gent
6 Y	S (14)	S (23)	S (30)	R (0)	S (25)	S (23)
518	R (0)	S (26)	S (30)	S (28)	S (25)	S (23)
3647	R (0)	ND	S (25)	ND	ND	S (24)
BW64	ND	ND	Presumed sensitive	ND	ND	ND

* S= Susceptible, R= resistant, (Zone Size in mm)

Table 4. Reactions in Volunteers During 6 Days
After Ingestion of 10^4 A. hydrophila Cells

Strain	6 Y				518
Patient Number	1	2	3	4	1 2 3 4
Number Stools Passed: *					
Formed	7	6	8	2	16 4 4 10
Soft	7	3	8	2	15 2 3 7
Watery	0	3	0	0	1 1 1 3
Days Strain Shed in Stool	0	0	0	0	0 1 0 0
	1	0	0	0	0 0 0 0

*During a 6 day hospital confinement

Table 5. Reactions in Volunteers During 5 Days
After Ingestion of $10^6 - 10^7$ A. hydrophila Cells

Strain	6Y				518				3647			
Patient Number	1	2	3	4	1	2	3	4	1	2	3	4
Number Stool Passed: *												
Formed	7	6	5	4	6	3	7	5	2	5	14	5
Soft	7	5	1	4	6	3	7	5	2	5	9	3
Watery	0	1	3	0	0	0	0	0	0	0	5	1
Days Strain Shed in Stool	0	0	1	0	0	0	0	0	0	0	0	1
	2	0	0	0	0	0	0	0	0	0	0	0

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Appendix A

Examination for Participating Students

Circle the one correct answer for each question.

1. The diarrhea bacteria given to students in this study is called:
 - A. Clostridium
 - B. Shigella
 - C. Salmonella
 - D. Aeromonas
2. Half of the students will receive a control strain. Which of the following describe the control strain:
 - A. A strain which doesn't possess disease-producing properties
 - B. A strain given in lower dose
 - C. A strain killed by heating
 - D. A strain of another bacteria
3. Which of the following is the reason to give baking soda first in this study:
 - A. To prevent heartburn
 - B. To neutralize stomach acid to help bacteria make it to the intestine
 - C. To add salt to diet to prevent dehydration
 - D. As a source of nourishment for the test strain
4. Which one of the following requirements is not part of the study?
 - A. Making 100% on this exam
 - B. Having the study explained twice
 - C. Being able to withdraw at any time and receiving treatment
 - D. Receiving an antibiotic to eliminate the bacteria
 - E. Being hospitalized for three weeks
5. Which one of the following tests is not part of the test?
 - A. Two blood samples
 - B. Bone marrow examination
 - C. Daily stool samples
 - D. Placing a tube in intestines (upper intestines and lower intestines) if diarrhea occurs
6. How will the diarrhea be treated if illness occurs?
 - A. Warm compresses to the abdomen
 - B. Intravenous fluids and antibiotics
 - C. Drugs to bind the intestine like Lomotil
 - D. Aspirin or Pepto-Bismol to prevent fluid loss

HELP STAMP OUT

TURISTA

HOW: Participate in a medical research study of the causes of traveler's diarrhea.

WHERE: Methodist Hospital

WHEN:

WHY: Learn about medical research.
Free physical examination.
Free room and board.
Cash reimbursement.

FOR MORE INFORMATION

Call: 792-4708

(Study reviewed and approved by the Committee for Protection of Human Subjects, The University of Texas Health Science Center at Houston)